



Trade Science Inc.

ISSN : 0974-7419

Volume 12 Issue 4

# Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 12(4) 2013 [133-140]

## Determination of betahistine hydrochloride using a sensitive fluorescent probe

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Received: 28<sup>th</sup> July, 2012 ; Accepted: 5<sup>th</sup> October, 2012

### ABSTRACT

The inclusion interaction of cucurbit[7]uril (CB[7]) with Betahistine Hydrochloride (BH) was studied using fluorescent probe titration method. Significant quenching of the fluorescence intensity of the CB[7]-palmatine (PAL) fluorescent probe was observed when BH was added, showing that BH was accommodated into the hydrophobic cavities of CB[7]. The hydrophobic interaction between CB[7] and BH mainly contributed to the formation of 1:1 type CB[7]-BH. The association constants of the complexes formed between the host and the guest were determined. The competing reaction and the supramolecular interaction mechanisms between the BH and PAL as they fight for occupancy of the CB[7] cavity were studied using spectrofluorimetry, <sup>1</sup>H NMR, and molecular modeling calculations.

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### KEYWORDS

Betahistine hydrochloride;  
Cucurbit[7]uril;  
Palmatine;  
Supramolecular interaction;  
Fluorescent probe.

### INTRODUCTION

Cucurbit[*n*]uril (CB[*n*], *n* = 5–8, 10) is a macrocyclic compound consisting of *n* glycoluril units connected by 2*n* methylene bridges<sup>[1–3]</sup>. The symmetrical CB[*n*] hosts resemble a hollow barrel with hydrophobic cavities and restrictive polar portals lined with ureido carbonyl groups<sup>[2,3]</sup>. These characteristics enable CB[*n*] to form significant stable complexes with a variety of guest molecules in aqueous solution. Various organic drugs and biologically relevant molecules have been encapsulated by CB[*n*]<sup>[4,5]</sup>. The formation of inclusion complexes often enhances or disturbs the photophysical and photochemical properties of the included guest molecules<sup>[6]</sup>. An increasing number of papers on different CB[*n*] host-guest interactions with a wide range of com-

pounds have been reported over the last few years<sup>[7–9]</sup>. However, little attention has been devoted to their fluorescent properties and potential analytical applications. Megyesi<sup>[10]</sup> discovered that the formation of an inclusion complex between CB[7] and berberine leads to improved fluorescence intensity. Li<sup>[11]</sup> studied the supramolecular interactions of CB[7]-isoquinoline alkaloid complex and its applications to the molecular recognition and determination of isoquinoline alkaloids and other drugs, owing to its excellent optical properties. It is just started that the interaction between the non-fluorescent drug and CB[*n*] has been studied by fluorescent probes titration<sup>[12]</sup>. Fluorescent probe system consisting of CB[7] and PAL fluorescent probe was successfully used for a variety of non-fluorescent drug determination because of its high sensitivity and selectivity<sup>[13]</sup>.

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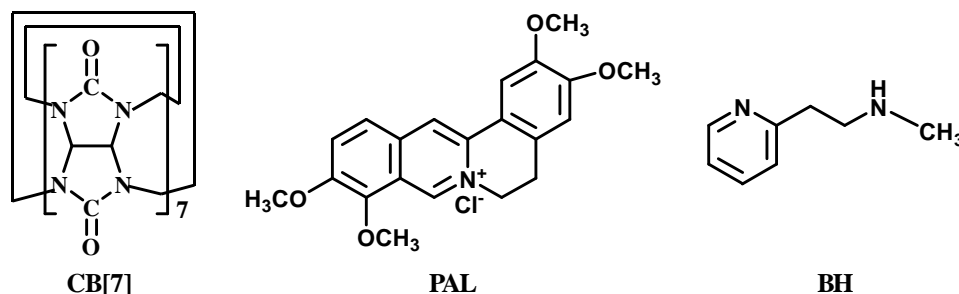


Figure 1 : The structures of CB[7], PAL, BH

Betahistine hydrochloride (BH) is a categories of vasodilatation. It has action of ectasia to the cardiovascular and cerebral vessels, especially to the arterial system. A number of assays have been reported for the determination of BH in biological and pharmaceutical samples, including HPLC<sup>[14]</sup> and capillary electrophoresis<sup>[15]</sup>. Spectrofluorometry is considered as the most convenient analytical technique in pharmaceutical analysis, owing to its inherent simplicity, high sensitivity, and availability in most quality-controlled and clinical laboratories<sup>[16]</sup>. Considering that aqueous solutions of BH have no native fluorescence, they cannot be directly determined through the normal fluorimetric method. Hence, the development of a fast, simple, and highly sensitive spectrofluorimetric method for the determination of BH in aqueous solution is highly desirable.

In our study, the supramolecular fluorescent probe system is based on the competition between the guest molecules and the probe molecules for the hydrophobic cavity of CB[7]. Such competition results in optical property changes. In the absence of appropriate guest species, the probe molecules partially reside in the hydrophobic cavity of the CB[7] and form stable inclusion complexes. However, the presence of organic analytes leads to the decomplexation of the probe molecules and to a concomitant decrease in fluorescence intensity. To our knowledge, the use of PAL as fluorescent probe for the determination of BH has not yet been reported. The proposed method in the current study is fast, simple, and highly-sensitive. The detection limits for BH is  $0.0042 \mu\text{g mL}^{-1}$ , making the proposed method more sensitive than any other method reported in the literature<sup>[17,18]</sup>. The proposed procedure was tested for the determination of drugs in their pharmaceutical dosage forms and in urine samples.

## EXPERIMENTAL

### Instruments

Fluorescence spectra and intensity measurements were obtained using a Hitachi F-4500 spectrofluorimeter equipped with a 150 W xenon lamp (Japan). The slit widths of both excitation and emission monochromators were set to 5 nm. The fluorescence spectra were recorded at a scan rate of  $1200 \text{ nm min}^{-1}$ . All measurements were performed using a standard 10 mm path-length quartz cell at  $25.0 \text{ }^\circ\text{C} \pm 0.5 \text{ }^\circ\text{C}$ . The pH values were measured using a pH-3 TC digital precision pH meter (Shanghai, China). <sup>1</sup>H NMR spectra were obtained using a Bruker DRX-600MHz spectrometer (Switzerland) in D<sub>2</sub>O.

### Reagent and chemicals

PAL and BH were obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) without further treatment. BH were dissolved in double-distilled water to prepare stock standard solutions of  $100 \mu\text{g mL}^{-1}$ . PAL was dissolved in double-distilled water to prepare stock solutions with final concentration of 1.0 mM. CB[7] was prepared and characterized according to reported procedure<sup>[19]</sup>. CB[7] stock solution of 1.0 mM was prepared by dissolving CB[7] in double-distilled water. Stock standard solutions were stable for several weeks at room temperature. Standard working solutions were prepared by diluting the stock standard solutions with double-distilled water before use. All other chemicals were of analytical reagent grade, and double-distilled water was used throughout the procedure.

### Experimental procedure

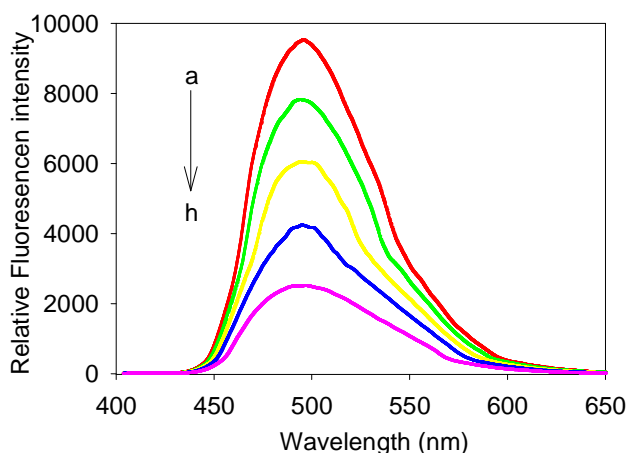
0.8 mL solution of 0.1 mM CB[7] was poured

into a 10 mL colorimetric flask, to which 0.8 mL of the 0.1 mM PAL solution and 1.0 mL of 0.01 M hydrochloric acid were also added. Suitable amounts of BH solution were then sequentially added to the flask. The mixture was diluted to volume with double-distilled water and shaken for 15 min at room temperature. The fluorescence intensity values of the solution ( $F_{\text{PAL-CB[7]-BH}}$ ) and the blank solution ( $F_{\text{PAL-CB[7]}}$ ) were measured at 495 nm using an excitation wavelength of 343 nm.

## RESULT AND DISCUSSION

### The fluorescence quenching of CB[7]-PAL probe by BH

The fluorescence quenching of CB[7]-PAL by BH. Significant quenching of fluorescence intensity of the CB[7]-PAL complex with the addition of BH was observed. The fluorescence spectra of the CB[7]-PAL complex, in the presence of different concentrations of BH, are shown in Figure 2, respectively. Fluorescence intensity decreased with increased BH concentration, which is likely due to the competition between BH and the PAL molecules for occupancy of the CB[7] cavity. Parts of the PAL molecule can be expelled from CB[7] cavity by the introduction of the BH, thereby reducing the fluorescence intensity of CB[7]-PAL because of the formation of a new inclusion complex between BH and CB[7].



**Figure 2 : Fluorescence spectra of CB[7]-PAL in the presence of BH in 1.0 mM HCl aqueous solution with  $\lambda_{\text{ex}} = 343$  nm. The concentrations of BH ( $\mu\text{g mL}^{-1}$ ): (a) 0; (b) 0.6; (c) 1.2; (d) 1.8; (e) 2.4  $C_{\text{CB[7]}} = 8.0. \mu\text{M}$ ,  $\text{BH}_{\text{AL}} = 9.0 \mu\text{M}$ .**

### Apparent association constant and stoichiometry

The addition of a nonfluorescent host (H) results in the formation of 1:1 host-guest inclusion complexes (H-G) which, in turn, enhances the fluorescence of the guest (G). The value of the enhanced fluorescence is dependent on the concentration of the added H, as shown in the following equation<sup>[19,20]</sup>.

$$F/F_0 = 1 + (F_\infty/F_0 - 1) \frac{[H]K}{(1 + [H]K)} \quad (1)$$

where  $F_0$  is the fluorescence intensity of G in the absence of H,  $F$  is the observed fluorescence intensity at each H concentration tested,  $F_\infty$  is the enhancement when 100% of G is complexed, and  $K$  is the equilibrium association constant for the 1:1 complexation.



$$K = \frac{[\text{H-G}]}{[\text{H}][\text{G}]} \quad (3)$$

The 1:1 complexation (and, hence, the applicability of Eq. 1) can be confirmed from the double reciprocal plot of  $1/(F/F_0 - 1)$  versus  $1/[\text{H}]$ . The plot will be linear if only 1:1 complexation occurs and will be non-linear if higher-order complexes also form.

In the interaction between CB[n] and PAL, the equilibrium reaction is as follows:



The equilibrium association constant is defined as follows:

$$K_{\text{CB}[n]\text{-PAL}} = \frac{[\text{CB}[n]\text{-PAL}]}{[\text{CB}[n]][\text{PAL}]} \quad (5)$$

Figure 3 showed the enhancement of PAL fluorescence as a function of added CB[7]. The inset shows the linear double reciprocal plot ( $r = 0.998$ ), confirming the 1:1 stoichiometry of the complex. The solid line shows the best fit of the data to Eq. 1 using the nonlinear least squares method. Four such trials were performed, yielding average values of  $K_{\text{CB[7]-PAL}} = (8.02 \pm 0.76) \times 10^5 \text{ M}^{-1}$  for the CB[7]-PAL equilibrium system.

In the interaction between CB[7] and BH, the equilibrium equation is as follows:



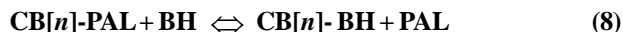
The equilibrium association constant is defined as

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follows:

$$K_{\text{CB}[7]\text{-BHT}} = \frac{[\text{CB}[7]\text{-BHT}]}{[\text{CB}[7]][\text{BHT}]} \quad (7)$$

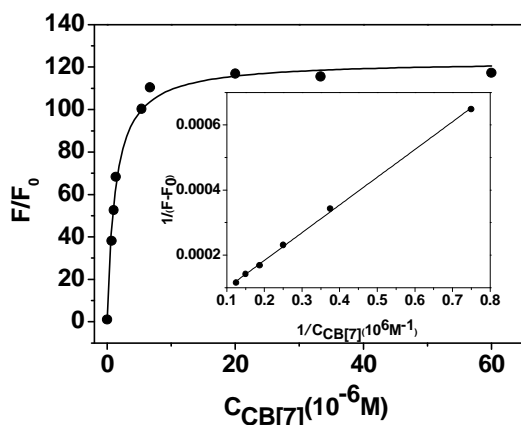
When BH was added to the aqueous solution of the CB[7]-PAL complex, equilibria (4) and (6) coexist in the solution. Thus, combining (4) and (6) gives the following equilibrium equation:



The equilibrium association constant is defined as follows:

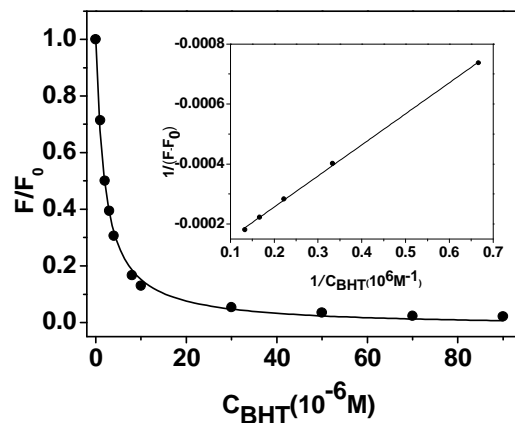
$$K = \frac{K_{\text{CB}[7]\text{-BHT}}}{K_{\text{CB}[7]\text{-PAL}}} = \frac{[\text{CB}[7]\text{-BHT}][\text{PAL}]}{[\text{CB}[7]\text{-PAL}][\text{BHT}]} \quad (9)$$

Wagner studied Eq. 1 and reported that it applies whether the fluorescence being measured is that of the guest or the host (i.e., they are interchangeable in Eqs. 2 and 3 because of the 1:1 complexation). In addition, Eq. 1 equally applies to the measurement of fluorescence quenching, as opposed to fluorescence enhancement<sup>21</sup>. The only difference is that the  $F/F_0$  values are less than 1.0 in fluorescence quenching. In the present study, the CB[7]-PAL complex was considered the host molecule. Thus, Eq. 1 can also be applied to the quenching of CB[7]-PAL complexes (host) fluorescence with the addition of BH (guest). Eq. 1 can be used to determine the equilibrium association constant  $K$  (Eq. 9) from the fluorescence titration data of  $F/F_0$  at a fixed CB[7]-PAL concentration as a function of BH concentration.



**Figure 3 :** Fluorescence enhancement of the fluorescence of a 2.0  $\mu\text{M}$  solution of PAL as a function of added CB[7] in 1.0 mM HCl aqueous solution with  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 343/495$  nm; the solid line shows the best fit of the data to Eq. 1. The inset shows the linear double reciprocal plot indicating 1:1 complexation.

Figure 4 showed the fluorescence quenching of CB[7]-PAL complexes with the addition of BH. The inset shows the linear double reciprocal plot ( $r = 0.999$ ), indicating the formation of a 1:1 host-guest inclusion complex between BH and CB[7]. The solid line shows the best fit of the data to Eq. 1. Four such trials were performed, yielding average values of  $K = (3.58 \pm 0.72) \times 10^5 \text{ M}^{-1}$  for the CB[7]-PAL + BH equilibrium system (Figure 7). The association constant value of  $K_{\text{CB}[n]\text{-BH}}$  (Eq. 7) can be calculated according to Eq. 9:  $K_{\text{CB}[7]\text{-BH}} = (2.83 \pm 0.49) \times 10^{11} \text{ M}^{-1}$ . These values are extremely large, indicating very strong host-guest interactions with excellent size and shape matches. A comparison of  $K_{\text{CB}[7]\text{-PAL}}$  with  $K_{\text{CB}[7]\text{-BH}}$  obviously results in  $K_{\text{CB}[7]\text{-BH}} \gg K_{\text{CB}[7]\text{-PAL}}$ . Thus, BH shows extremely stronger binding with CB[7] than PAL. Accordingly, considering the thermodynamic factor only, the PAL molecule can be expelled from the CB[7] cavities by the tested drug molecules.



**Figure 4 :** Fluorescence suppression of the fluorescence of CB[7]-PAL as a function of added BH in 1.0 mM HCl aqueous solution with  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 343/495$  nm; the solid line shows the best fit of the data to Eq. 1. The inset shows the linear double reciprocal plot indicating 1:1 complexation.  $C_{\text{CB}[7]} = 2.0 \mu\text{M}$ ,  $C_{\text{PAL}} = 2.25 \mu\text{M}$ .

### Effect of PAL concentration on the fluorescence intensity of the CB[7]-PAL complex

The effect of varying PAL concentrations on the fluorescence intensity of the CB[7]-PAL complex was studied. The concentration of PAL was varied from 1.0  $\mu\text{M}$  to 10.0  $\mu\text{M}$  for the CB[7]-PAL system. The fluorescence intensity of the CB[7]-PAL complex was gradually enhanced as PAL concentration increased until it reached the maximum inclusion equilibrium at CB[7]

saturation. In the present paper, PAL served as a fluorescent probe; thus, determining the proper concentration was crucial. If the PAL concentration is too low, the sensitivity of the probe will also be low. Conversely, a very high concentration may not help determine the optimum detection limit of the analyte. Taking everything into consideration, the optimal PAL concentration was 9.0  $\mu\text{M}$  for CB[7]-PAL complex.

### Influence of pH

The effect of pH on  $\Delta F$  was studied over the pH range of 1.0 to 12.0. The results indicate that  $\Delta F$  is maximum and almost constant in the pH range of 1.0 to 7.0. However,  $\Delta F$  significantly decreased with further increases in pH. The reason is that alkali cations are readily coordinated to the carbonyl-fringed portals of CB[7] in alkaline medium. Binding of the alkali cation lowers the rate constant of the ingress of organic guests ingression. Results show that the presence of sodium salts in alkaline medium lowers not only the equilibrium constant of PAL binding with CB[7] but also the fluorescence quantum yield. Hence, using hydrochloric acid, the pH was adjusted to 3.0, which was the desired pH for all subsequent experiments.

### Influence of temperature and reaction time

The effect of temperature on  $\Delta F$  was examined within 10 °C to 80 °C. All formed complexes were stable up to 35 °C. Above 35 °C, the fluorescence intensity greatly decreased due to the dissociation of the complexes at high temperatures. Hence, all subsequent measurements were performed at room temperature.

In addition,  $\Delta F$  reached a maximum within 10 min after the BH was added and remained constant for at least 5 h. Hence, the standard reaction condition was set to room temperature for 10 min.

### The response mechanism of the fluorescent probe.

PAL exhibits weak fluorescence emission in aqueous solution because the isoquinoline and the substituted benzene rings in PAL are not on the same plane. This configuration prevents a conjugated system from being formed. When CB[7] was added into the aqueous solution of PAL, the electrostatic attraction between the positive charge of the heterocyclic nitrogen of the PAL and the high electron density of the carbonyl oxygens of the macrocycle was induced. The apolar

part of PAL can penetrate into the host cavity. Inside the cavity, the degree of freedom of motion of the PAL molecule is reduced, thus also reducing the probability of radiationless transition. At the same time, the cavity can shield the excited single state of PAL from probable quenching processes that usually occur in aqueous solutions.

Molecular modeling calculations were optimized at the B3LYP/6-31G(d) level of density functional theory<sup>[22]</sup> using the Gaussian 03 program. Results confirmed the partial inclusion of PAL in the hydrophobic cavity of CB[7]. The partial immersion of PAL in the hydrophobic cavity of CB[7] reduces interaction with water. This state results in a less polar microenvironment which, in turn, leads to fluorescence enhancement.

When BH was added to the host-guest system of CB[7]-PAL, the PAL and BH competed to occupy the CB[7] cavity. Some parts of the PAL molecule were expelled from the CB[7] cavity with the introduction of the BH. The energy-minimized structures of CB[7]-BH complex are shown in Figure 5, the methyl is located in the vicinity of a carbonyl-laced portal. The photochemical property of PAL is strongly dependent on its local microenvironment. The addition of the BH caused PAL to lose its protection in the CB[7] hydrophobic cavity, thus resulting in reduced PAL fluorescence intensity. The competitive inclusion model is shown in Figure 5.

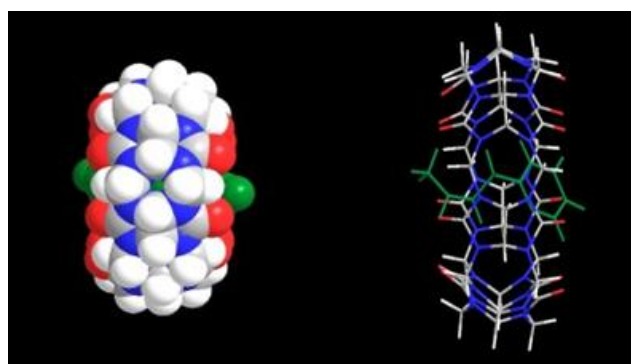


Figure 5: Energy-minimized structure of CB[7]-BH complexes in the ground state using balls and tubes for the rendering of atoms. Color codes: BH, green; CB[7], oxygen, red; nitrogen, blue; carbon, gray; hydrogen, white.

The formation of CB[7]-PAL complex had been confirmed by  $^1\text{H}$  NMR spectroscopy<sup>[12]</sup>. The results of the  $^1\text{H}$  NMR experiments are consistent with the theoretical structures of the complex. Compared with the proton resonances of the unbound BH molecules, the

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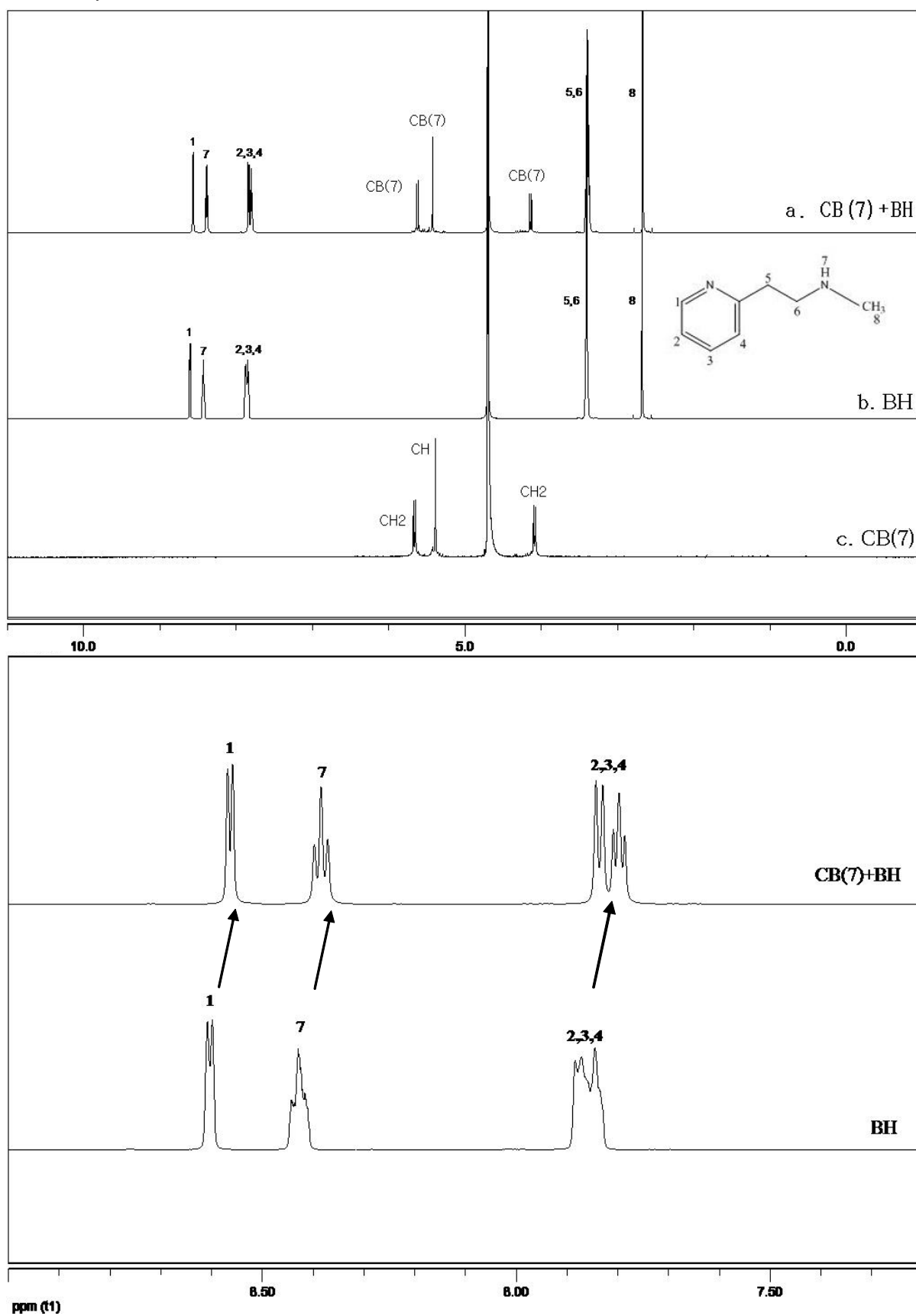


Figure 6 : <sup>1</sup>H NMR spectra (600 MHz) of CB[7] (a), BH (b), CB[7]-BH complex (c)

signals of H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub>, H<sub>4</sub>, H<sub>7</sub> protons of the bound BH significantly shifted upfield. This behavior is characteristic of this part of the molecule encapsulated in the CB[7] cavity<sup>[23]</sup>. The chemical shift of the H<sub>5</sub>, H<sub>6</sub>, H<sub>8</sub> protons is practically unchanged, which indicates negligible interaction with CB[7]. These findings indicate that the PAL molecules were expelled from the CB[7] cavity due to the addition of BH. These results are consistent with the foregoing discussion.

In summary, combination of hydrophobic interaction of the cavity of CB[7] as well as ion-dipole interaction between the carbonyl portal of CB[7] and N<sup>+</sup> ion (of PAL, and BH) leads to the formation of host-guest complex. Because of the excellent size and shape matches, BH bound with CB[7] more tightly than PAL.

### Effect of interfering substances

Prior to the application of the proposed spectrofluorimetric method to real samples, the effect of commonly used tablet excipients on the determination of 0.2 μg mL<sup>-1</sup> of BH was studied under optimum experimental conditions. A 3000-fold mass in excess of each substance over BH was first tested. When interference occurred, the ratio was progressively reduced until the interference ceased. The tolerance limit was defined as the ratio of the concentration of the foreign species to that of BH which resulted in an error of less than ± 5%. The results are shown in TABLE 1. The determination was obviously free of interference from the usual excipients. During the testing of real samples for the determination of pharmaceutical preparations, no other interfering substances having similar structure with BH in excipients were observed. Thus, no background interference was present, indicating good selectivity in the method used to test the drug in both raw

TABLE 1: Effect of interference (tolerance error ± 5.0%)

Tolerance ratio in mass	Interference
3000	Starch, glucose, sucrose, lactose, sorbitol, mannitol, boracic acid,
2000	Methyl cellulose, Cl <sup>-</sup> , PO <sub>4</sub> <sup>3-</sup> ,
1500	Glycin,
1000	Sodium hydroxymethyl cellulose, gum acacia power, tryptophan,
500	Sodium carboxymethyl cellulose,
100	NH <sub>4</sub> <sup>+</sup> , Na <sup>+</sup> , K <sup>+</sup> , ascorbic acid
50	Mg <sup>2+</sup> , Zn <sup>2+</sup> , Ca <sup>2+</sup> , Fe <sup>2</sup>
0.3	Alanine, cysteine, valine, cystine, phenylalanine,

material and dosage forms.

### Calibration graph and sensitivity

Under the optimum experimental conditions described above, a linear relationship between Δ*F* intensity and the concentration of BH was obtained in the range of 0.011–2.6 μg mL<sup>-1</sup>. A correlation coefficient of 0.9997 and a detection limit of 0.0042 μg mL<sup>-1</sup> were also determined. The linear regression equation obtained was Δ*F* = 2392.2*C* + 15.653. The proposed method proved to have higher sensitivity than any other spectral method for determining BH reported in the literature.

The proposed fluorescent probe was applied in the determination of BH in pharmaceutical preparations. Ten tablets of BH were carefully pulverized. From this powder, an equivalent of 10 mg of BH was accurately weighed, placed inside a 100 mL calibrated flask, dissolved in 20 mL water, and swirled and sonicated for 3 min. The resulting solution was diluted to volume with water. The first 10 mL of the filtrate was discarded, after which 10 mL of the remaining filtered sample solution

TABLE 2 : Analytical results and recovery tests of BH in pharmaceutical formulation

Drugs	Label claim (mg/grain)	Fluorescent probe method			Reference method	
		Found (mg/grain)	Equivalent nominal content (%) ± S.D. <sup>a</sup>	Recovery(%) ± S.D. <sup>a</sup>	Found (mg/grain)	Equivalent nominal content (%) ± S.D. <sup>a</sup>
BH <sup>b</sup>	10	9.93 ( <i>t</i> , 1.54; <i>F</i> , 2.92)	99.3 ± 0.54	99.65 ± 0.59	9.853	98.53 ± 0.68
BH <sup>c</sup>	5	4.88 ( <i>t</i> , 1.65; <i>F</i> , 3.18)	97.60 ± 0.72	97.82 ± 0.78	4.847	96.94 ± 0.82

<sup>a</sup> Average of five determination. (The tabulated values of *t* and *F* at the 95% confidence limit are *t* = 2.31 and *F* = 6.39.)

<sup>b</sup> BH Tablets (Tianjin Lisheng Pharmaceutical Co., Ltd, Tianjin, China)

<sup>c</sup> BH Tablets (Shanxi Tongda Pharmaceutical Co., Ltd, Shanxi, China)

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was diluted to 100 mL with double-distilled water. Further dilutions were performed to obtain the sample solutions using the same testing methods as described in experimental procedure. The results are presented in TABLE 2. The concentrations of BH determined using the proposed fluorescent probe are consistent with the obtained through methods found in the literature. The relative standard deviations obtained from the proposed method were less than 1.00%. Moreover, the *t*- and *F*-tests showed that the proposed method had better precision and accuracy compared with the method. The recoveries were in the range of 97.82% to 99.65%.

### CONCLUSION

The new fluorescent probe system for CB[7]-PAL complexes was devised for the determination of analytes. Considering the significant quenching observed in the fluorescence intensity of CB[7]-PAL in the presence of BH, the spectrofluorimetric method developed was of high sensitivity and selectivity for the determination of BH in aqueous solution. The proposed method is more sensitive than the other spectral methods reported in the literature. Moreover, the method had been successfully applied to the determination of BH in its pharmaceutical dosage forms. This method can also be used in a fluorescence sensor for the detection of non-fluorescent or weakly fluorescent substances. Related studies are in progress in our laboratory. At the same time, the interaction models of the supramolecular complexes were established through theoretical calculations. The interaction mechanism between the fluorescent probe and BH was confirmed via the <sup>1</sup>H NMR spectrum. The association constants of the complexes formed between the host and the guest were calculated.

### ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China (No. 21171110) and the Research Fund for the Doctoral Program of Higher Education of China (No.20091404110001). Helpful suggestions by anonymous referees are also gratefully acknowledged.

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